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Recent studies have shown that NSAIDS such as aspirin reduce the incidence of human cancers by inhibiting the enzyme Cyclooxygenase (COX), which plays a key role in arachidonic acid metabolism. It is now known that COX exists in at leas two isoforms, term COX-1 and COX-2. Of these, COX-2 has also been found to be constitutively expressed in a number of tumor tissues, including breast. The purpose of out study to develop new COX-2 inhibitors that can be sued in breast cancer therapy. We have exploited the structural differences between the two COX enzymes to develop specific inhibitors of COX-2 and have identified three classes of novel COX-2 inhibitors that possess tumor growth inhibitory activity. Some of these compounds inhibit growth of both COX-2 positive as well as COX-2 negative tumor cell lines, suggesting that these compounds might target another protein that plays an important role n the growth of tumor cells. These studies suggest that these compounds may play an important role as an anti-cancer and chemopreventive agents.

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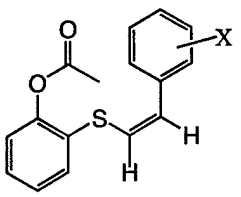
Title: Novel COX-2 Inhibitor for Breast Cancer Therapy

Introduction: Cyclooxygenase-1 and 2 (COX-1 and COX-2) enzymes catalyze the biosynthesis of prostaglandin H2 by converting arachadonic acid to prostaglandins (1). Recent studies have shown that high levels of COX-2 are expressed in a large percentage of tumors, including those of the breast. In mice, transgenic expression of COX-2 in the mammary gland has been found to result in highly malignant and metastatic breast tumors after repeated rounds of pregnancy, firmly establishing a role for this gene in the genesis of breast cancer (2-5). In addition, constitutive over-expression of the COX-2 gene has been observed in greater than 50% of ductal carcinomas *in situ* and in several highly metastatic, estrogen receptor (ER)-negative breast tumor cell lines (6,7). These studies provide compelling evidence to support the involvement of COX-2 in the development of breast cancer and it is therefore reasonable to conclude that drugs that target COX-2 activity may be of significant importance in the treatment of this disease. In addition, epidemiological studies have shown that the use of NSAIDs lower the incidence of certain tumors in humans, including those of the breast (2-5, 8-10). Animal studies using COX-2-specific NSAIDS have confirmed these findings. Celecoxib, when administered daily in the diet of rats, inhibited the development of 7,12-dimethylbenz(a)anthracene (DMBA)- induced mammary tumors and induced tumor regression in animals (8,9). These studies demonstrate that COX-2 specific NSAIDS act as both anti-carcinogenic and anti-neoplastic agents with respect to breast cancer. More importantly, the fact that they are devoid of any side effects underscores the need to develop new and improved agents to treat this disease.

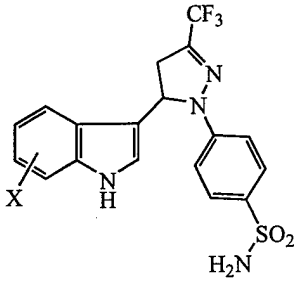
Body: To achieve the first aim, we synthesized a series of novel compounds aimed at identifying the most potent COX-2 inhibitor that can be used in breast cancer therapy. These compounds belong to three classes; (i) Derivatives of SKU-46 (18010-18050, 18100, 18110, 18130, and 18140; (ii) Derivatives with a Pyrazoline backbone (9250, 9250A, 9250B, 9260, 9270, 9280, 9290, 9300, 9310, 9310A, 9310B, 9320, 9330, 9340, 9360, 9390, 9400, 9410, 9420, 9440, 9450, 9460, 9470, 9480, 9490 and 9500) and (iii) Derivatives with a Hydrazone backbone (26010, 26020, 26030, 26040, 26050, 26060, 26120, 26120, 26130, 26130, 26140, 26150 and 26160). The structures of these compounds are shown in Table 1. In addition, celecoxib was used as a standard.

Table 1.

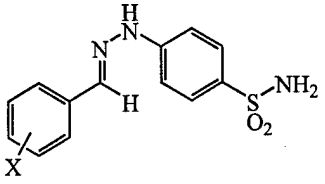
ON 18XX0



ON 09XX0



ON 26XX0



Analogs Synthesized

Compound	X	Compound	X
ON 18010	H	ON 09470	5-NO2
ON 18020	4-F	ON 09480	5-COOH
ON 18030	2-F	ON 09490	7-NO2
ON 18040	4-Cl	ON 09500	7-NH2
ON 18050	4-Br	ON 26010	4-$CH_3C_6H_4$
ON 18100	2-OCOCH3	ON26020	4-FC_6H_4
ON 18110	4-OCH3	ON26030	4-ClC_6H_4
ON 18130	2,4-F2	ON26040	4-BrC_6H_4
ON 18140	4-CF3	ON26050	3-Indoyl
ON 09250	H	ON26060	$C_6H_5CH_2$
ON 09250A	H (-)	ON26120	4-$(CH_3O)_3C_6H_4$
ON 09250B	H (+)	ON26130	2,Cl,4-FC_6H_4
ON 09270	5-F	ON26140	3,4-$F_2C_6H_4$
ON 09280	5-Cl	ON26150	3-F,4-$NO_2C_6H_4$
ON 09290	6-F	ON26160	4-$NO_2C_6H_4$
ON 09300	6-Cl	ON26170	3-FC_6H_4
ON 09310	7-Cl		
ON 09310A	7-Cl (-)		
ON 09310B	7-Cl (+)		
ON 09320	5-CN		
ON 09330	6-Br		
ON 09340	5-NH2		
ON 09360	4-NH2		
ON 09400	6-CN		
ON 09410	6-NO2		
ON 09420	2-CH3,6-Cl		
ON 09440	SO2CH3		
ON 09450	4-OCH3		
ON 09460	5-NH2		

The inhibitory effect of these three series of drugs against COX-2 (ovine) enzyme was analyzed using a COX inhibitory screening assay kit (Cayman chemicals, MI) (12). This assay directly measures PGE_{2a}, produced by stannous chloride reduction of COX-derived $PGEH_2$, via enzyme immunoassay (EIA). This COX assay is more accurate and reliable than an assay based on peroxidase inhibition (Gierse, et al., 1999). In brief, stock solutions of COX-2 inhibitors were prepared in DMSO. Different concentrations (0.001 μM to 100 μM) of inhibitors were made in a reaction buffer (Tris-HCl (0.1M, pH 8.0)) containing 5mM EDTA, 2mM phenol and 1μM hematin. Known concentrations of COX-2 inhibitors were pre-incubated with highly purified ovine COX-2 (6 units) enzyme, without substrate, at room temperature for 60 minutes. After the incubation period, 100μM arachidonic acid substrate was added to the reaction mixture. The reaction was stopped after 2 minutes by adding 50μl 1M HCl, followed by the addition of 100μl saturated stannous chloride. The final product, PGE_{2a}, was measured using EIA. The level of inhibition was calculated as the percentage of COX-2 activity compared to the total activity of COX-2 and the IC50 values were obtained according to the manufacturers instructions. In this study, IC_{50} values for each compound were determined from two independent experiments.

Effect of SKU-46, 9250 and 26000 series compounds against COX-2 activity: IC_{50} values obtained for all the compounds were above 10μM except that 18100 recorded 3μM. The IC_{50} value obtained for celecoxib was 1.7 μM. IC_{50} values determined for the hydrazone compound lies between 3.75μM and 100μM. Of the pyrazoline derivatives, compounds such as 9250A and 9310A showed more than two fold higher inhibition than celecoxib in COX-2 inhibition assays, whereas most of the other pyrazoline derivatives showed moderate effect compared to celecoxib. These results suggest that 18100 and 9310 are most potent inhibitors of COX-2 and might be useful for cancert therapy and prevention.

Effect of SKU-46, 9250 and 26000 series compounds on Breast tumor cell viability: To assess the anti-tumor effects of these compounds, we next incubated COX-2 positive and COX-2 negative tumor cell lines with all of the compounds shown in Table 1 and determined the cell viability 96 hours post-treatment. The results of viability experiments using COX-2 negative cells showed that the GI_{50} values of 18050 and celecoxib are similar. However, the GI_{50} values determined for compounds 18010, 18040, 18100, 18110 and 18140, with COX-2 positive cells,were similar to the value measured for celecoxib. This indicates that the SKU-46 series compounds kill cancer cells in a COX-2 dependent manner. Among the hydrazone derivatives, 26150 compounds recorded a GI_{50} value of 7μM, whereas the GI_{50} of celecxoib was 13.1μM for COX-2 negative cells. However, in COX-2 positive cells, GI_{50} values measured for most of the compounds were lower than the value measured for celecoxib (15.9 μM).

Growth Inhibition of COX-2 positive Breat Tumor Cells by ON09250. To determine the effect of ON09250 on the growth of COX-2 positive tumor cells, we utilized an end point dose response assay. For this assay, we plated $1.0x10^5$ COX-2 positive tumor cells in six well dishes in the appropriate growth medium. Increasing concentrations of either by ON09250 or ON09300 (an analog of ON09250) or Celecoxib were added to the cells after 24 hrs. The number of viable cells was determined by trypan blue exclusion 96 hours following the addition of the drug. The dose response showed that ON09250 induced 50% reduction in the number of viable cells (compared to vehicle-treated controls) at a concentration (GI_{50}) of approximately 20 μM while the GI_{50} of Celecoxib was 45μM. To further confirm these results, we next examined the ability of by ON09250 and Celecoxib to inhibit the growth of MDA-MB-231 breast tumor cells in soft agar. In these studies, ON09250 showed complete inhibition of colony formation at a concentration of 10μM, while Celecoxib required a concentration of 40μM for a similar result (data not shown). Both the ON09250 and Celecoxib-treated cells were found to be positive in Tunel assays, suggesting the activation of apoptotic pathways by both drugs.

ON09250 induces growth inhibition and cell death of both COX-2 positive and COX-2 negative tumor cell lines. In view of recent studies which indicate that Celecoxib inhibits the growth of both COX-2-positive and negative cell lines, we examined the effect of ON09250 on the growth of several COX-2 positive and negative cell lines. These studies show that ON09250 induces growth inhibition and death of both COX-2 positive and negative cell lines.

Induction of Death Receptor-5 (DR-5) by ON09250. Based on studies by Huang et al (13), we examined that ability of Celecoxib, ON09250 and its chloro analog, 9310 and Sulindac sulfide to induce DR-5 receptors following incubation for 24 hrs in the presence of 10μM concentration of each of these compounds. The results of these studies show that indeed ON09250 and 9310 are potent inducers of DR-5 and their superior activity against tumor cells might be related to this activity.

Key Research Accomplishments: We have identified three classes of novel COX-2 inhibitors that possess tumor growth inhibitory activity. Some of these compounds inhibit growth of both COX-2 positive as well as COX-2 negative tumor cell lines, suggesting that these compounds might target another protein that plays an important role in the gowth of tumor cells.

Reportable Outcomes: None

Conclusions: The identification of a role for COX-2 in breast tumor growth necessitates the development of specific inhibitors for this enzyme. For cancer therapy, it is necessary to develop new agents that possess growth inhibitory and pro-apoptotic properties that are more efficacious than the present group of drugs used for the treatment of inflammation. Our results to-date show that the three classes of compounds developed by us show specific inhibition of COX-2 and have growth inhibitory activity at low concentrations against breast tumor cells. These studies suggest that these compounds may play an important role as an anti-cancer and chemopreventive agents.

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